Some observations on the development of Colletotrichum lindemuthianum in artificial cultures.

GEO. F. ATKINSON.

WITH PLATE XXII.

Several times during the summer of 1893 I attempted to obtain a pure culture of the bean anthracnose for the purpose of noting its behavior in artificial media. Dilution cultures were attempted in the ordinary agar-agar peptone broth, from material recently collected but which had dried. None of the spores germinated. Thinking that the nutrient agar might be an unfavorable medium for their growth, cell cultures were started in water to test the vitality of the spores. The spores here likewise failed to germinate for me. This seemed surprising, for related forms of *Gloeosporium* and *Colletotrichum* of quite a number of species have never failed to germinate promptly even after several months drying.

Some of the material from which the attempts were first made was used in the dilutions within three days after picking the fresh pods of the bean, which contained the spores of the fungus in great numbers, and they had not been dry for more than twenty-four hours. Still they failed to germinate for me. Several other attempts were made during the autumn of the same year with like failures.

During February of the following year, 1894, preparations were made for another attempt at obtaining a pure culture of the fungus. Since the fungus is perennial in matured beans which are affected, it was planned to obtain diseased but mature and dry beans, and then grow them in order to obtain in the laboratory freshly developed spores. Accordingly requests were made from several leading seedsmen for badly anthracnosed beans for the purpose of obtaining material for cultures. Some very fine specimens were received of what is known as Wardwell's kidney wax. The beans presented an unsightly appearance, being stained various shades of yellow and fuliginous, some of them also possessing depressed spots where the fungus was more deeply seated. In one specimen which presented nearly one entire side in a badly diseased condition, there were also several pustules which appeared

as if spores were present in a dormant condition. This bean was placed in a moist chamber to induce the fresh development of spores. March 7, 1894 (a few days later), the pustules were considerably larger and the material was examined. Fresh spores were present in great numbers and dilution cultures for the separation of the fungus were started on the same day. The room temperature was rather low, and on the following day none of the spores had germinated though some which were seen had swollen to some extent and refringent granules were appearing. Culture number one was then left in a warmer room and on the following day the spores were germinating and their study in this condition was then made. The germ tubes are very large, equalling or even in some cases exceeding the diameter of the spore. The first tubes usually arise near the ends of the spore and are generally directed at a greater or less angle from the axial line of the spore. The refringent granules are quite numerous and large so that the protoplasm presents a very coarsely granular appearance. In the homogeneous protoplasm vacuoles also soon appear but they are at first quite indistinct. The threads for a short distance from the spore describe a sinuous course and branch in an irregularly monopodial fashion, at the same time other threads arise from the spore so that a small radiating colony is soon developed. Quite soon however on the margin of the small colony the threads frequently present a dichotomous appearance. This in some cases is brought about by a perfect dichotomy of the thread but very frequently and perhaps in a majority of cases there arises a branch just behind the growing end of the thread which very soon overtakes the primary thread, and the influence of its origin so close to the end of the thread causes the growing end of the same to be diverted so that the appearance of dichotomy is the result. On the very young colony only a few of the threads on the margin present this appearance, but soon all of the threads partake in this dichotomous branching and very frequently it occurs successively in rapid sequence on the same thread, so that a plumose or brush shaped tuft is produced. The various branches of this tuft lie nearly parallel and quite close together. At this time and indeed very soon after germination the vacuoles in the protoplasm become quite large and prominent. Also at the ends on the plumose branches very short lateral branches now

arise, several on one side, and the end of the thread is frequently curved to the same side. Upon these short branches are developed the dark bodies which appear in certain of the anthracnoses sometimes called secondary spores.

On March 10th from dilution culture no. 2 small colonies of the fungus were transplanted to vetch stems in culture tubes for pure cultures. In two days a very minute growth appeared at the points of the transplantings as fine radiating white threads. In a few days more the spots of the central point of growth became black by the darkening of the threads while the advancing margin of the weft continued white. On the 14th spores were found to be developing in considerable numbers. By the 16th areas varying from 1 to 2^{cm} in length on the stems were occupied by the very black and thin stroma of the fungus. Except where the threads had reached the liquid in the bottom of the culture tube there was no considerable development of white fungus threads. In the liquid however quite a profuse growth took place. From the surface of the dark stroma in some cases a scanty growth of whitish threads arose for a few millimeters from the surface. The dark stroma itself was roughened by the development of irregular tuberculate prominences.

April 20th pure dilution cultures were started to obtain the spores in different stages for the study of germination and the following development stages, more especially that these might be recorded in photomicrographs while the organisms were *in situ*.

The dilutions were made in Petri dishes. On the following day, the 21st, the cultures were examined and no spores were found germinating, though they were present in numbers and there was no difficulty experienced in finding them in the cultures.

The cultures were examined again on the 22nd, and while the spores did not appear as if they were dead there were none germinating. On the 23d a few of the spores were found to be germinating.

On the 24th at 5 P. M. additional cultures were made in Van Tieghem cells in the following way. The cells were prepared and the cover glasses sterilized by passing several times through the flame and then placed under a bell jar to protect them from gravitating germs while the culture material was being placed on them. In order to have a large number of

spores in a small space about one-half cc. of liquid agar was prepared in culture tubes and these inoculated by transplanting a considerable quantity of the mycelium and attached spores from the culture on vetch stems. This would probably assure a large number of spores in the liquid. With a looped sterilized platinum needle a small quantity of the inoculated liquid was lifted from the tube and allowed to spread upon the center of the cover glass, only one transfer being made, and the liquid thus was held in a thin layer until solidification took place. Many of the spores were thus in close contact with the glass, and in germination would be nearly in the same plane.

During the afternoon of the following day the spores began germinating in the cell culture and one of the spores with four germ tubes was photographed (fig. 1). This same spore was photographed on the following day, twenty hours later, and is shown in fig. 2. In all cases unless the spore is very short and nearly oval a septum appears at the time of germination, forming two cells, and at the point of the septum the spore becomes constricted even soon after germinating. The cell culture now became contaminated with bacteria and further growth was impossible. Even at the time of photographing no. 2 the bacteria at this place were quite numerous, and the flocculent matter which clouds one portion of a thread and the margins of others is a mass of bacteria (fig. 2).

The cultures in the Petri dishes were now examined again, dilution no. 1 first. A few colonies were visible to the unaided eye as irregular stellate patches. This examination showed that the spores after germination had continued to increase in size for some time so that they became several times larger than when germination first takes place. One of these was photographed with a magnification of about 500 diameters (fig. 4). With this magnification only the central portion of the colony could be shown. The threads radiating from the spore turn in various directions so that it is not possible to bring them all in the same focal plane. The spore was the point which was focussed upon and a few of the threads are in the same focal plane and show the proportionate diameter, the septation and size of the cells. At this time the highly refringent granules which appear at the time of the germination of the spore and are then comparatively

small, are now much larger and quite strongly differentiated from the hyaline contents. They are quite numerous in the enlarged spore and are also present to a less degree in the threads.

At the same time it was observed that the very large majority of spores in the same culture, which had failed to germinate at the time the first ones germinated, had continued to increase in size, were once septate, strongly constricted at the point of the septum and were richly charged with large highly refringent granules. It fact but for the germination of the first spores there could not be determined any difference. One of these is shown at fig. 6. The margin was a trifle out of the focal plane so that the wall presents a heavier line than should be the case. In culture dilution no. 1 none of these spores germinated, but in culture dilution no. 2 nearly all of them began germinating on the 25th and on the 26th several of these were photographed to show the different results which follow. Fig. 7 shows one with only the ordinary germ tubes, fig. 8 is developing basidia directly from the spore and bearing several spores, others had but few germ tubes terminated by the oval, dark bodies. It thus seems that these spores which do not readily at first germinate become for a short time places for the storage of reserve material, and later germinate. Whether they would under conditions giving an abundance of room produce colonies like the first ones has not been determined, for in the present culture they were too close together for this result.

In plate culture no. 2 the older and stellate colonies developed a compact stroma at the center which bore numerous spores with a slight roseate or flesh colored tinge. On April 28th four cultures on sterilized bean stems were made of this dilution culture in order to study the behavior of these normally developed spores in comparison with those which were late in germinating and did not develop in this culture many spores. Two of these cultures on bean stems were made by transplanting spores from the normally developed colonies, and two were made by transplanting some of the agar containing the second type of spores which were late in germinating.

May 7th a culture, using numerous spores, was made in a Petri dish during the afternoon. This culture was made by pouring a small quantity of agar containing numerous spores

over the surface of previously solidified agar in the plate, thus securing numbers of spores in a thin plane at the surface of the medium. On the following morning eighteen hours after sowing, the culture was examined and many of the spores were found to be germinating, the temperature being quite favorable to quick germination for this species. One group of spores was selected for a photomicrograph (fig. 11), containing three spores with young germ tubes and two spores not yet germinated. One of the spores germinating shows plainly the division of the spore into two cells at the time. Twenty-four hours later considerable growth had taken place. The preceding day when the first photomicrograph was taken, a cover glass was placed over a portion of the culture in order to prevent the moisture from the surface of the agar from condensing on the objective when the strong reflected rays of the sun should be mirrored through the culture during the exposure.

From former experiences it was found that very little growth was made after having once shut out the access of oxygen by placing a cover glass over a number of the spores. In this case the growth was surprising for it was quite considerable, though not so much as that of spores not thus covered. Perhaps this considerable growth compared with the very little or none in other experiences was due to the peculiar way in which this culture was made. A photomicrograph of the growth of one spore was made at this time (forty-two hours after sowing) and is shown in fig. 12. The spore itself at the center of the colony can be seen from its greater diameter than that of any of the threads. It is also quite strongly constricted at the center which is brought about by the rapid enlargement or swelling of the spore. In order to bring certain of the threads into strong focus which it was desired to reproduce with their characteristic features in detail the spore was thrown slightly out of focal plane, and the septum in the spore is not well seen and the vacuoles not distinct. In certain of the threads however the vacuoles and septa are distinctly shown. The peculiar dichotomous branching of the hypha which frequently occurs in this species is shown in two of the threads. A photograph taken three hours later of this same colony is represented in fig. 13. In one of the dichotomous divisions one fork has considerably outgrown the other. The growth of the spores which were not covered by the cover

glass was by this time considerably in advance of that of the ones used to illustrate these features. In this culture as well as in those previously studied, many of the spores did not germinate at first, but manifested their activity by absorbing nutrient material and assimilating it from the medium, thus increasing in size and in the richness of the granules. This is also accompanied by the formation of the cross wall making two cells in most cases and also in a greater or lesser constriction of the spore at the middle. These spores begin to germinate at varying intervals so that the process of germination is going on for several days or even for a week. Forty-eight hours after sowing the spores, two of them having recently germinated were photographed (fig. 14). One spore possesses two very short germ tubes, one at each end; the other spore which was slightly out of the focal plane has not only a germ tube from each end but some from the side as well, and all but one of the threads are quite long and flexuous. Sixty-six hours after sowing two other spores were photographed (fig. 15). Here the spores are of considerable size, a result of the continuous swelling, and the septum at the strongly constricted central portion can easily be seen.

Cornell University.

Explanation of Plate XXII.

Figures 1 to 15 are photomicrographs of the living organism in the nutrient agar where it was growing. The objects were therefore unstained.

In figures 16 and 17 are represented, natural size, the form of the mature colonies of two plate cultures.